Genetics of Reproduction and Regulation of Honeybee (*Apis mellifera* L.) Social Behavior

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Abstract

Honeybees form complex societies with a division of labor for reproduction, nutrition, nest construction and maintenance, and defense. How does it evolve? Tasks performed by worker honeybees are distributed in time and space. There is no central control over behavior and there is no central genome on which selection can act and effect adaptive change. For 22 years, we have been addressing these questions by selecting on a single social trait associated with nutrition: the amount of surplus pollen (a source of protein) that is stored in the combs of the nest. Forty-two generations of selection have revealed changes at biological levels extending from the society down to the level of the gene. We show how we constructed this vertical understanding of social evolution using behavioral and anatomical analyses, physiology, genetic mapping, and gene knockdowns. We map out the phenotypic and genetic architectures of food storage and foraging behavior and show how they are linked through broad epistasis and pleiotropy affecting a reproductive regulatory network that influences foraging behavior. This is remarkable because worker honeybees have reduced reproductive organs and are normally sterile; however, the reproductive regulatory network has been co-opted for behavioral division of labor.

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BACKGROUND

The honeybee is the only social insect that has been successfully bred for social traits with clear documentation of selective improvement. The most successful and best-documented case demonstrates how selection on a social phenotype resulted in changes in the reproductive anatomy and regulatory network effecting changes in social behavior (7, 40, 67). Reproductive mechanisms and social behavior have dominated hymenopteran genetics since their origin more than 160 years ago. In 1845, Johannes Dzierson, a parish priest in Salesia (now a part of Poland) published a paper in which he hypothesized that male honeybees are derived from unfertilized eggs (26). They have mothers but no fathers. Female honeybees are derived from fertilized eggs, making them biparental. His hypothesis, the first proposed mechanism for sex determination, was met with great skepticism from his beekeeping colleagues but was confirmed in 1856 by Carl Th. von Siebold (114) whose microscopic studies showed that male-destined eggs did not contain sperm. This was 50 years before the discovery of sex chromosomes (121). Subsequent cytological studies by Nachstheim (60) confirmed that male honeybees had a single set of chromosomes (haploid) whereas females had two sets (diploid). Recently, researchers identified and characterized a single gene, *complementary sex determining* (*csd*), that provides the primary signal for haplodiploid sex determination in honeybees (14, 33).

Dzierson's discovery did not go unnoticed by Gregory Mendel. He wanted to use Dzierson's discovery of haplodiploidy to confirm his theory of inheritance within an animal system. The basic idea was to produce queens that were hybrids of two different geographical races that differed in body color: black and yellow. Then, because males have no fathers, the two color types should segregate equally in male progeny of the queen. However, Mendel ran into an immediate difficulty: Honeybee queens mate with many males (106) while they fly through the air, often kilometers away from the hive (nest); thus, he was unable to control which males were successful (48, 64, 70). The inability to control mating also thwarted Mendel's lifelong ambition to breed the social trait of making bees that were better honey producers.

It took nearly 150 years before the problem of controlling mating was solved through the efforts of Loyd Watson, W.J. Nolan, Otto Mackensen, and Harry Laidlaw (52). Their efforts spanned three decades, but by the 1950s instrumental insemination technology was perfected, and researchers were able to control honeybee matings. Honeybee genetics was then pursued in earnest. Hellmich et al. (40) provide a carefully executed and documented case of successful artificial selection. They successfully selected lines of honeybees that stored more (high lines) and less (low lines) pollen. Page & Fondrk (67) used the social phenotype, selection, and breeding methods of Hellmich and colleagues to begin an investigation of the genetic and phenotypic architectures of a social trait across levels of biological organization. Others joined this endeavor, forming a collaborative community.

HONEYBEE NATURAL HISTORY

A honeybee society (colony) typically consists of a single queen that has mated with a large number of males (estimates vary but often more than 10) and stored their sperm for her normal egg-laying life of 1–2 years. Ten to 40,000 facultatively sterile worker adults, all female, perform all the tasks required for colony growth, maintenance, and defense. Anywhere from zero to several hundred males may be present, depending on the time of year. Worker adults progress through a behavioral-developmental process: When young, they perform tasks such as cleaning, feeding larvae, constructing comb, processing food, etc., within the nest and then forage for food, water, and a building material called propolis outside the nest. This transition usually takes place in the second or third week of life. The queen lays almost all the eggs [on rare occasions, workers may lay eggs (66)], and at any given time, more than 30,000 eggs,

larvae, and pupae may be present (for a review of honeybee biology, see Reference 122).

A SOCIAL PHENOTYPE

Pollen hoarding involves the storage of surplus pollen in the wax combs of the honeybee nest. It is a complex behavior involving thousands of socially interacting individuals. The nest of a honeybee colony is organized spatially such that the eggs, larvae, and pupae (brood) are placed three dimensionally in the center of the nest toward the bottom. A thin envelope of pollen surrounds the brood. Honey is stored to the sides and the above the brood. Bees that are less than approximately two weeks old feed proteinaceous glandular secretions to developing larvae. The secretions are derived from proteins contained in the pollen consumed by the nurse bees. The quantity of pollen stored by a colony in a nest is regulated by the foragers (18, 24, 25, 30, 82, 112). When stored pollen is added to a colony, pollen-foraging activity is reduced until the excess is consumed. When stored pollen is removed, pollen-foraging activity increases until the loss is replaced. Stored pollen inhibits pollen-foraging behavior in individuals, whereas brood pheromone, a blend of hydrocarbons produced and secreted by larvae to their external cuticle, stimulates pollen foraging (77). The response of foragers to the combination of these stimuli results in the regulation of stored pollen. Therefore, the amount of pollen stored in the comb is an easily measured phenotype that results from the combined efforts of the larvae, pollen-consuming nurse bees, and pollen-collecting foragers.

PHENOTYPIC ARCHITECTURE

Page & Fondrk (67) selected for a single trait—high and low pollen hoarding—for more than 40 generations spanning more than 22 years (R.E. Page & M.K. Fondrk, unpublished results). The result was two strains of bees that differ dramatically in the amount of pollen they store. The purpose of this experiment was to document changes at different levels of biological organization, from colony-level traits to the genome, and then map the architecture of the pollen-hoarding phenotype. **Table 1** provides details of the phenotypic architecture along with a comprehensive list of supporting citations. In the sections below, we provide brief descriptions of the important consequences of colony-level selection.

Colony Level

A significant colony-level response to selection was seen in a single generation (67). By the fifth generation, the high strain had six times more stored pollen than did the low strain. Currently, the high strain hoards more than 12 times more pollen (R.E. Page & M.K. Fondrk, unpublished data). The proportion of the foragers collecting pollen was increased in the high strain. This was a result of a reallocation of foragers from nectar- to pollen-foraging biases in the highs and the inverse in the lows. The total number of foragers did not change. Differences in stored pollen were not a consequence of differential queen or brood cues and signals (67).

Individual Level

Compared with low-strain foragers, high-strain foragers initiate foraging earlier in life, are more likely to return with a load of pollen, and collect larger pollen loads. The inverse is automatically true regarding nectar because the number of foragers is not different between high- and low-strain colonies. Total load size is restricted for foragers; therefore, bees that collect larger pollen loads collect smaller nectar loads (46, 68, 91). Foraging bias can be expressed as the proportion of the total foraging load that is allocated to pollen: (pollen load)/(pollen load + nectar load).

When foraging for liquids, high-strain bees are more likely to return with nectar with lower sugar concentrations and are more likely to be water foragers. This is probably a consequence of their greater response sensitivity to water and sugar solutions (see the Sensory-Motor Response and Learning section

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Table 1 Phenotypic traits of high- and low-strain bees for different levels of organization[a]

Level	Trait	Effect	Reference(s)
Colony	Stored pollen	H > L	(67)
	Stored honey	None	(67)
	Brood area	None	(67)
	Number of bees	None	(67)
	Number of foragers	None	(67)
	Number of pollen foragers	H > L	(34, 67, 71)
	Number of nectar foragers	L > H	(34, 67, 71)
	Proportion pollen foragers	H > L	(34, 67, 71)
	Queen cues	None	(71)
	Larval cues	None	(71)
Individual	Pollen load	H > L	(28, 29, 34, 67, 71, 74–76, 78, 91)
	Nectar load	L > H	
	Nectar sugar concentration	Usually L > H	(65, 76, 78; cf. 91)
	Water load	H > L	(65, 74)
	Floral preference	None	(34)
	Age of foraging onset	L > H	(76, 78, 89)
	Response to pollen foraging stimuli	H > L	(29, 74, 76, 111)
	Dance for pollen	H > L	(115)
	Scout for pollen	H > L	(23)
	Attend pollen dances	H > L	(C. Dreller & R.E Page, unpublished data)
Anatomy	Body mass	L > H	(54, 55)
	Ovariole number	H > L	(1, 35, 54, 55, 88)
Development	Time from egg to adult	H > L	(8)
	Juvenile hormone in larval stage 5	H > L	(8)
	Juvenile hormone in pupal stage and young adults	Different dynamics	(8, 101)
	Ecdysteroids in pupal stage and young adults	Different dynamics	(8)
	Vitellogenin in young-adult workers	H > L	(3, 4, 8)
	Vitellogenin/juvenile hormone dual-repression regulation	H = yes L = no	(3, 47)
	Ecdysteroid production by worker ovaries	H > L	(8)
	Sensitivity to colony rearing environment	H > L	(54, 55, 78)
	Sensitivity to in vitro rearing environment	L > H	(55)
	Body mass: ovariole number (reared in colonies)	Strain and rearing environment interactions	(55; O. Kaftanoglu & R.E. Page, unpublished data) (note that panels in Figure 1 are reversed)
Sensory motor	Sensitivity to sugar and water	H > L	(65, 74, 78, 79, 96, 97, 110)
	Sensitivity to light	H > L	(110)
	Locomotor activity	H > L	(43, 86)
Associative learning	Tactile and odor	H > L	(53, 96, 97)

(*Continued*)

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Table 1 (*Continued*)

Level	Trait	Effect	Reference(s)
Nonassociative learning	Sensitization	H > L	(V. Kolavenuu & R.E. Page, unpublished manuscript)
	Habituation	L > H	(V. Kolavenuu & R.E. Page, unpublished manuscript)
Neurobiochemistry	Protein kinase A	H > L	(44)
	Protein kinase C	H > L	(44)
	Octopamine	H = L	(101)
	Serotonin	H = L	(101)
	Dopamine	H = L	(101)

[a]Some traits differed between high- and low-strain (H and L, respectively) bees as a consequence of the effects of selection.

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below). Nectar-load size positively correlates with nectar sugar concentration (68, 78, 91, 105), and pollen-load size negatively correlates with nectar load. Therefore, sensitivity to sugar is a fundamental component of honeybee forage-loading algorithms (105).

Selection for pollen hoarding did not affect floral preferences: Highs and lows apparently visit the same flowers, at least in the environments where they were tested (34). However, social signaling during recruitment to food sources was affected. Some foragers are scouts; they fly out from the nest without being recruited by others and find new resources. They return and perform recruitment dances to advertise the quality and location of the food sources they discover (103, 113). High-strain bees are more likely to find and recruit others to pollen sources when they are scouts (23). They dance more for pollen sources (115), and as potential recruits, they spend more time attending pollen dances (C. Dreller & R.E. Page, unpublished data). High-strain bees are more responsive to pollen-foraging stimuli, brood pheromone, and stored pollen. They also demonstrate greater changes in response to changing cues and signals (29, 77, 79, 111).

Reproductive Anatomy and Development

Several developmental differences were noted between high- and low-strain workers. Some of these are likely developmental signatures of colony-level selection. High-strain bees require approximately 18 h longer to develop from egg to adult. Normal development is about 21 days. Compared with low-strain workers, high-strain workers have slightly lower body mass and more ovarioles. Per data from Linksvayer et al. (55), high- and low-strain bees had wet weight body masses of 101.8 and 105.4 mg ($F_{1249} = 6.17$; $P < 0.05$) and 11.2 and 9.2 ovarioles ($F_{1243} = 16.71$; $P < 0.0001$), respectively. Worker honeybees normally have fewer than approximately 25–30 total ovarioles, whereas queens have more than 300 (1, 35, 54, 55, 88, 90). In early larval development, workers and queens have the same high number of developing ovarioles. However, worker ovaries undergo programmed cell death beginning in approximately the fifth (final) larval instar (99, 100). Queen ovaries are rescued from apoptosis by elevated blood titers of juvenile hormone (JH), an insect-growth regulator. Worker larvae have reduced amounts of JH during development. High-strain larvae have significantly more JH circulating compared with low-strain larvae and, consequently, more ovarioles complete development (8). We believe this is a signature of colony selection because of the subsequent effects of worker ovaries on foraging bias (see below). JH and ecdysteroids work together to interact dynamically throughout larval and adult reproductive development (39). High- and low-strain workers differ in the dynamics of

these two hormones throughout development; however, the functional significance of these traits remains unknown. It is interesting to note that males of the high and low strains also differ phenotypically in their adult maturation rates, indicated by differences in the timing of the onset of mating flight behavior (86).

In insects, ovaries and fat body interact in a reproductive regulatory network (7, 39). Key signaling elements are JH produced in the *corpora allata* (paired glands associated with the brain), ecdysteroids produced in the ovary, and vitellogenin (Vg) produced in the fat body. When signaled, the fat body produces Vg, a lipoprotein that is taken into the ovaries and incorporated into the eggs. High-strain ovaries produce more ecdysteroid and, prior to foraging, their fat body produces more Vg in young workers (8, 109). Vg inhibits JH production and JH inhibits Vg production in a double-repressor feedback loop (6). Together, they regulate the onset of foraging. Vg titers in young adults affect sensory response systems and foraging behavior later in life (47, 61, 109), as explained further below. Low-strain bees have lost the double-repressor regulation of JH and Vg (3, 47), another developmental signature of colony-level selection on a social trait.

Sensory-Motor Response and Learning

High-strain bees are more sensitive to water and to lower concentrations of sugar solutions when tested using the proboscis-extension response test (51). Sugar serves as a reward in classical associative learning studies, and high-strain bees perform better on the learning tests, a consequence of their having placed a higher value on the sugar reward to which they are more sensitive (95). Sucrose sensitivity of newly emerged bees is linked to age of onset of foraging and foraging bias later in life (72, 73, 75).

The Pollen-Hoarding Syndrome

Comparison of differences found between high- and low-strain workers with wild type (not part of the selected populations) reveals suites of traits that covary (**Table 2**) and confirms the role of colony-level selection in shaping the differences observed in high and low strains. The role of the ovaries is apparent in that wild-type bees with more ovarioles produce more Vg, forage earlier in life, are more sensitive to dilute-sugar solutions, and demonstrate a pollen-foraging bias. Newly emerged bees that are more sensitive to sucrose solutions and water forage earlier in life, collecting larger pollen loads; as nectar foragers, they also collect more dilute nectar. These are the anatomical and behavioral traits that define the high and low pollen-hoarding strains that were a consequence of selection for pollen hoarding and which we have called the pollen-hoarding syndrome (43). We assume a simple model: Selection on the colony-level trait affects ovary size, thereby affecting Vg levels that, in turn, affect the sensory system as measured by the response to sugar and, consequently, the loading of the forager with pollen and nectar (**Figure 1**). Effects were measured on these components and are presented as the proportion of the variance in combined groups of high- and low-strain bees, or between groups of wild-type bees, that can be explained by their genotypes, the statistical R^2. Effects were strongest for the social trait selected. Effects on ovary size were nearly as strong, followed by sensitivity to sugar, whereas direct effects of genotype on individual foraging behavior were relatively weak, though statistically significant.

The pollen-hoarding syndrome was further confirmed through genomic mapping of quantitative trait loci (QTLs) associated with many of the traits. The genetic architecture revealed an overlapping network of QTLs showing broad epistasis and pleiotropy for the suite of traits. We identified candidate genes from the QTL regions and tested for messenger (m)RNA expression differences between bees of the high and low pollen-hoarding strains. We then developed RNA interference (RNAi) knockdown methods to study their effects (see below).

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Table 2 Statistically significant correlations ($P < 0.05$) between traits related to the pollen-hoarding syndrome for high- and low-strain bees and for wild-type (i.e., not selected for pollen hoarding) bees

Correlation	Stock	Reference(s)
Ovarioles: vitellogenin	WT	(1, 4, 8, 109)
Ovarioles: foraging onset	WT	(1, 116, 118)
Ovarioles: foraging bias	H, L; WT, AHB, Apis cerana	(3, 87, 104 105, 116, 118)
Ovarioles: nectar-sugar concentration	H, L; WT	(1, 105, 116)
Ovarioles: sucrose response	WT	(5)
Ovarioles: *HR46* expression	H, L; WT	(116–118)
Ovarioles: *PDK1* expression	H, L, H/L backcross	(116)
Ovarioles: *TYR* expression	H, L; WT	(117)
Vitellogenin: foraging onset	H,L; WT	(47, 61)
Vitellogenin: nectar load	H,L; WT	(47, 61)
Vitellogenin: sucrose response	WT	(5, 109)
Sucrose response: light response	H,L; WT	(27, 85, 110)
Sucrose response: associative learning	H,L; WT	(95–97)
Sucrose response: nonassociative learning	WT	(94)
Sucrose response: locomotor activity	WT	(43)
Sucrose response: foraging onset	WT, AHB	(72, 75)
Sucrose response: foraging bias	WT, AHB	(65, 72, 73, 75, 96, 104)
Sucrose response: nectar concentration	WT	(72, 73, 75, 104)

Abbreviations: AHB, Africanized honeybee; H, high-strain bee; L, low-strain bee; WT, wild type.

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GENETIC ARCHITECTURE

QTL studies using the high and low pollen-hoarding strains have revealed a highly epistatic and pleiotropic genomic network that maps onto the phenotypic architecture of the pollen-hoarding syndrome. Until the mid-1990s, complex behavior in a natural context, such as the pollen-hoarding syndrome, was considered by most as too stochastic to be studied genetically and only one behavioral gene, the *for* gene in *Drosophila*, had been identified in a natural context (21). However, the strong initial selection response in the divergent pollen-hoarding strains encouraged researchers to pursue genetic studies of these behavioral differences, especially because the focal trait of selection, pollen hoarding, is a highly regulated colony-level trait (30, 40, 71). Colony-level traits result from the joint action of thousands of individuals, reducing the stochastic variance (12) that is inherent to many behavioral and other complex phenotypes.

Pollen Hoarding

Thus, after two generations of divergent selection and preliminary genetic analyses (71), the first genetic-mapping study of social behavior in honeybees focused on the composite trait of pollen hoarding at the colony level. The study used 38 colonies derived from backcrosses of interstrain hybrid males to supersister queens (69) of the high pollen-hoarding selection line (46). Despite this relatively small sample size, one significant and one suggestive QTL (*pln1* and *pln2*) were identified through genotypic analysis of the recombinant haploid fathers that sired all workers in the respective colonies (46). Although the linkage map was sufficiently saturated, further QTLs could not be excluded and the identified QTLs could not be localized

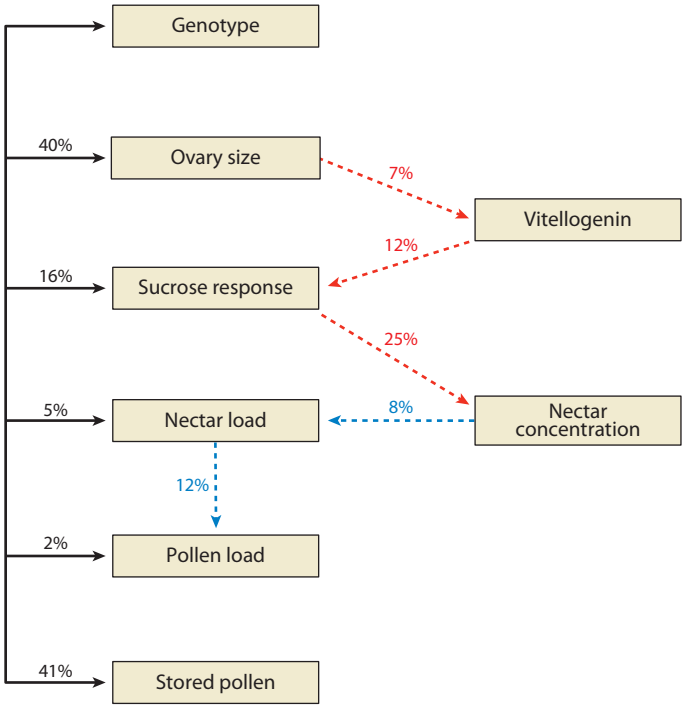

Figure 1

The R^2 values are presented as the percent of the total variance explained by regressing the variable shown at the head of the arrow against the variable at the base. All R^2 were statistically significant ($P < 0.05$). The relationship between genotype and stored pollen was estimated from unpublished breeding records by R.E. Page and M.K. Fondrk: Solid black lines indicate comparisons of high- and low-strain bees, red dashed lines represent wild-type bees, and dashed-dotted blue lines indicate wild-type bees and bees from the high and low strains. Figure based on data from several studies (1, 5, 73, 76, 109; also see figure 2 in Reference 2).

owing to the anonymous nature of the randomly amplified polymorphic DNA (RAPD) markers that were used (46). Attesting to their true Mendelian nature, however, two RAPD markers linked to the two *pln* QTLs could be scored three generations later to reconfirm the two QTLs by association with individual foraging behavior (46). Later, these RAPD markers were sequenced to convert them to genome site-specific sequence-tagged site (STS) markers.

Foraging Behavior

In addition to further confirmation of these existing QTLs, a third and fourth QTL for foraging choices were subsequently discovered (68, 91). The first of these studies used a cross between Africanized and European honeybees to remap the colony-level trait termed pollen hoarding, thus verifying *pln2* in this independent cross (68). The same study also remapped pollen hoarding in the high and low strains after outcrossing in the sixth generation, discovering a new significant QTL (*pln3*). After a second outcross, another backcross showed that allelic variation at *pln* QTLs affected the foraging choices of workers. *pln3* affected nectar-load size and nectar concentration of nectar foragers as well as pollen-load size of pollen foragers, whereas *pln2* affected pollen-load size of pollen foragers, which demonstrated pleiotropy of these QTLs across social and individual behavior (68).

The third QTL study of the foraging phenotype focused exclusively on individual foraging behavior of interstrain backcross-derived workers during their first foraging trip. It used amplified fragment length polymorphism (AFLP) markers for genome coverage in conjunction with the available STS markers to investigate the effects of *pln1–3* and the genome region near the candidate *amfor* gene (91). Unlike previous studies, reciprocal backcrosses to the pollen-hoarding strains [(H × L) × H and (H × L) × L] were studied. Furthermore, interactions between genotype markers were included in the analysis, implicating all four loci in significant multiway interactions (91). Direct effects were detected for *pln2* and a marker located in the *amfor* region, which was called *pln4* (45). In addition, two statistically significant and several new suggestive QTLs influencing worker foraging behavior were found (91). However, these QTLs lacked independent confirmation and genomic localization, two important requirements for follow-up studies; thus, they were not investigated further.

With the assembly and annotation of the honeybee genome (20), several STS markers were used to localize *pln1–4* in the genome and identify positional candidate genes (45). This analysis suggested the insulin-insulin-like signaling (IIS) pathway was involved because genes associated with this pathway were

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Table 3 The *pln* QTLs contain a significant number of genes associated with IIS[a]

QTL	ILS-associated genes	Total # of candidate genes	Physical size of QTL interval
pln1	PIG-P, bazooka	18	3390 kb
pln2	PIP5K, HR46	59	2106 kb
pln3	PI3K II, PDK1	32	1484 kb
pln4	IRS	4	131 kb

[a]As with QTLs, sequence data should be repeated. The high and low strains are currently being sequenced to verify the positional candidate genes in this table.
Abbreviations: IIS, insulin-insulin-like signaling; ILS, insulin-like signaling; IRS, insulin receptor substrate; QTL, quantitative trait locus. See Reference 45.

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significantly overrepresented in all four *pln* QTLs (45), including several central components to the IIS pathway (**Table 3**). Numerous subsequent studies of candidate-gene expression and repression have since confirmed the involvement of the IIS pathway in the pollen-hoarding syndrome (see below).

Sucrose Responsiveness and Age of Onset of Foraging

Owing to the phenotypic associations of the pollen-hoarding syndrome, the genetic architecture of other behavioral phenotypes was predicted to overlap with the genetic architecture of foraging behavior and pollen hoarding. The first two traits investigated were the age of first foraging (AFF) of workers and the sucrose responsiveness of newly emerged workers and drones. High and low strain bees showed significant differences and both traits were influenced by *pln* QTLs, particularly *pln1* (84, 89). The influence of *pln1* on sucrose responsiveness was detected in workers and drones (84), confirming the association of the pollen hoarding syndrome to male behavioral phenotypes. Additional, two-way interaction effects between the remaining *pln* QTLs were detected in the workers but not in drones (84). No new QTLs for sucrose responsiveness were discovered in the high backcross, but in the low backcross and the male mapping population two and one new QTLs were discovered, respectively (84).

The AFF of honeybee workers represents a textbook example of a complex life-history trait. Significant genetic differentiation exists between multiple natural populations (16, 72) and between the high and low pollen-hoarding strains (76). Initially, two reciprocal backcrosses of these strains were analyzed using more than 400 AFLP markers, revealing four QTLs (*aff1–4*) in addition to a direct effect of *pln1* (89). After the publication of the genome sequence, three *aff* QTLs (*aff2–4*) were localized and one (*aff1*) was rejected by a study that combined AFLP-marker sequencing, combined single-nucleotide polymorphisms and AFLP mapping, and microsatellite genotyping (83). No formal analysis was performed to link these QTLs to IIS, but the positional candidates near *aff* QTLs include homologs of genes that are involved in metabolic regulation (e.g., *amontillado*) (81) and several kinases, such as *PKC*, which varies in expression between high- and low-strain workers (44), and the MAP-kinase *ERK7*, which influences numerous physiological processes including reproduction. Functional genetic studies have provided corroborative evidence for some of these genes (e.g., *ERK7*) and the general involvement of reproductive hormones in the regulation of social behavior (120).

Ovary Size in Workers

Based on the finding of the phenotypic association between worker ovary size and other aspects of the pollen-hoarding syndrome (**Table 2**), two QTL studies were conducted on the genetic architecture of worker ovary size (35, 56, 88). These studies relied on a set

of single-nucleotide polymorphism markers, filling the remaining linkage gaps with additional microsatellite markers. This marker strategy allowed for even genome coverage and immediate localization of the genetic effects. Three significant QTLs were detected in reciprocal backcrosses between the high and low pollen-hoarding strains (88). A similar crossing scheme of selected Africanized and European sources resulted in strongly transgressive phenotypes in the Africanized backcrosses (56). QTL analysis of the two crosses demonstrating the most extreme transgressive phenotypes resulted in three significant QTLs (35).

Previously identified behavioral QTLs showed significant effects on ovary size in both studies of the genetic architecture of ovary size. Among the *pln* QTLs, *pln2* and *pln3* exhibited additive effects in the high pollen-hoarding backcross (116). Effects of *pln1* and *pln2* were identified (35) in the parallel backcrosses to the Africanized parent, whereas the effects of *pln3* were suggestive. In these Africanized × European crosses, the *aff* QTLs were also investigated and effects of *aff2* and *aff4* detected (35). In fact, the strongest ovary-size QTL coincided with *aff2* and the third strongest QTL overlaid *pln1* in this study. Two particularly interesting candidate genes in the ovary-size QTL intervals are long, noncoding RNAs that exhibit some of the most significant expression differences between the developing ovaries of workers and queens (42). Thus, widespread genetic overlap between worker ovary size and social behavior was found, confirming the central prediction of genetic cosegregation of social behavior and reproductive traits made by the reproductive ground-plan hypothesis (35, 116).

To link the associations between the worker ovary and social behavior to hormonal dynamics, an additional QTL mapping study simultaneously investigated the ovary size of workers and their JH titer in response to *vitellogenin* knockdown (see below) in a backcross of an interstrain hybrid queen to a high pollen-hoarding drone. This experiment relied on restriction-site-associated DNA sequencing (RAD-tag markers) (11), which can be generated rapidly in great numbers through high-throughput sequencing. As predicted, considerable overlap with previously identified QTLs was found: Markers near all six previously mapped ovary-size QTLs showed significant effects on ovary size, and two of these QTLs (on chromosome 2 and 3) also coincided with the strongest genetic effects on the workers' JH titers. Because one of these ovary-size QTLs corresponds to *aff2* and another to *pln1*, the association of these behavioral QTLs to ovary size was further confirmed. In addition, the *pln3* genotype influenced the JH titer, and *pln4* exhibited an effect on ovary size (K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript).

In sum, we have identified a network of interacting, pleiotropic genetic elements that underlie the central aspects of the pollen-hoarding syndrome (**Table 4**). This network partially overlaps between traits and is presumably incomplete because it explains only some of the total genetic variation. Furthermore, our body of work has revealed that the effects of particular QTLs strongly depend on the genetic background. Nevertheless, single, segregating factors with considerable effects have been identified and the majority of the localized QTLs have proven robust because their effects on the pollen-hoarding trait network have been confirmed in additional study populations. This result is particularly remarkable in honeybees because the consequences of inbreeding on sex determination do not allow production of isogenic lines (14), and honeybees have an exceptionally high recombination rate (13). The general importance of the main QTLs is emphasized by the overlapping results between selected-strain crosses and population crosses. However, we have not performed general population association studies of any QTLs or candidate gene to assess its importance in population-wide variation. Follow-up studies have instead focused on expression and repression studies, particularly of the IIS genes in the *pln* QTLs, as described in the following section.

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Table 4 Mapped QTL for pollen hoarding, sucrose responsiveness, foraging behavior, age of foraging onset, and worker ovary size[a]

QTL (chromonsome:Mb) trait	Map population	Effect and interactions	Reference(s)
		***pln1* (13:3.5)**	
Pollen hoarding	HBC	Direct	(46)
Pollen-load size	HBC	Direct × *pln2* × *pln3*	(46) (91)
Nectar-load size	HBC	Direct	(46)
Pollen proportion	LBC	× *pln3* × *pln2* × *pln3* × *pln4*	(91)
Nectar concentration	LBC	× *pln2* × *pln3* × *pln4*	(91)
Sucrose responsiveness	HBC, LBC, HXL hybrid males	Direct	(84)
AFF	HBC, LBC	Direct × *pln3* × *pln2* × *pln3*	(89)
Worker ovary size	ABC, HBC	Direct	(35; K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***pln2* (1:16.5)**	
Pollen hoarding	HBC, EBC	Direct	(46, 68)
Pollen-load size	HBC	Direct × *pln4* × *pln1* × *pln3* × *pln3* × *pln4*	(46, 68, 91)
Nectar-load size	HBC	Direct × *pln4*	(46) (91)
Pollen proportion	HBC, LBC	Direct × *pln4* × *pln1* × *pln3* × *pln4*	(91)
Nectar concentration	HBC, LBC	Direct × *pln1* × *pln3* × *pln4*	(46, 91)
Sucrose responsiveness	HBC	× *pln3*	(84)
AFF	LBC	× *pln1* × *pln3*	(89)
Worker ovary size	HBC	Direct	(116)
Worker ovary size	ABC	Direct	(35)
		***pln3* (1:9.2)**	
Pollen hoarding	HBC	Direct	(68)
Nectar-load size	HBC	Direct	(68)
Pollen-load size	HBC	Direct × *pln1* × *pln2* × *pln2* × *pln4*	(68) (91)
Pollen proportion	LBC	× *pln1* × *pln1* × *pln2* × *pln4*	(91)

(*Continued*)

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Table 4 (*Continued*)

QTL (chromonsome:Mb) trait	Map population	Effect and interactions	Reference(s)
Nectar concentration	HBC, LBC	Direct × *pln*4 × *pln*1 × *pln*2 × *pln*4	(68) (91)
Sucrose responsiveness	HBC, LBC	× *pln*2 × *pln*4	(84)
AFF	LBC	× *pln*1 × *pln*1 × *pln*2	(89)
Worker ovary size	HBC	Direct	(116)
JH response to Vg-RNAi	HBC	Direct	(K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***pln4* (13:9.0)**	
Nectar-load size	HBC	× *pln*2	(91)
Pollen-load size	HBC	× *pln*2 × *pln*2 × *pln*3	(91)
Pollen proportion	HBC, LBC	Direct × *pln*2 × *pln*1 × *pln*2 × *pln*3	(91)
Nectar concentration	HBC, LBC	Direct × *pln*3 × *pln*1 × *pln*2 × *pln*3	(91)
Sucrose responsiveness	HBC, LBC	Direct × *pln*3	(84)
Worker ovary size	HBC	Direct	(K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***aff2* (11:13.1)**	
AFF	HBC	Direct	(83, 89)
Worker ovary size	ABC	Direct	(35)
		***aff3* (4:9.1)**	
AFF	LBC	Direct	(83, 89)
		***aff4* (5:8.8)**	
AFF	LBC	Direct	(83, 89)
Worker ovary size	ABC	Direct	(35)
		***per1* (??)**	
Sucrose responsiveness	LBC	Direct	(84)
		***wos1* (3:13.1)**	
Worker ovary size	HBC	Direct	(88; K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
JH response to Vg-RNAi	HBC	Direct	(K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***wos2* (2:10.7)**	
Worker ovary size	HBC	Direct	(88; K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)

(*Continued*)

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Table 4 (*Continued*)

QTL (chromonsome:Mb) trait	Map population	Effect and interactions	Reference(s)
JH response to Vg-RNAi	HBC	Direct	(K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***wos3* (4:1.8)**	
Worker ovary size	LBC LBC	Direct	(88; K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***wos4* (11:10.7)**	
Worker ovary size	ABC HBC	Direct	(35; K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)
		***wos5* (6:14.2)**	
Worker ovary size	ABC HBC	Direct	(35; K.E. Ihle, O. Rueppell, R.E. Page & G.V. Amdam, unpublished manuscript)

[a]Crosses for map populations are as follows: ABC, Africanized backcross; EBC, European backcross; HBC, high-strain backcross; HXL, hybrid cross; LBC, low-strain backcross. For each QTL, effects are shown as direct or in interaction with other QTLs. Additional abbreviations: AFF, age of first foraging; JH, juvenile hormone; QTL, quantitative trait locus; RNAi, RNA interference; Vg, vitellogenin.

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CANDIDATE GENES

QTL mapping and the honeybee genome annotation provided several candidate genes involved in IIS and hormonal-response signaling in the pollen-hoarding QTL regions (**Table 3**). In addition, *pln2* contains a receptor for a neuromodulator that affects sucrose sensitivity (98), the reproductive states of workers (92, 93, 107), and the onset of foraging (102), all components of the pollen-hoarding syndrome. We looked at gene-expression patterns of six candidate genes in the fat body, brain, and ovaries of high- and low-strain workers of different ages and found significant differences in expression for *Tyr* (*pln2*), *PDK1* (*pln3*), and *HR46* (*pln2*), making them targets for further investigation. Three candidate genes did not show differential expression: *PAR3* (*pln1*), *P13K* (*pln3*), and *IRS* (*pln4*). However, *IRS* demonstrated a trend toward elevated expression in the high-strain worker fat body, and this difference was significant in a later study (118). Association studies using wild-type bees subsequently showed that, depending on the number of ovarioles, PDK1 and HR46 covary in expression in the fat body (116), suggesting that differential ecdysteroid signaling, as a consequence of ovary size (8), affects fat body expression of those genes. *PDK1* is a kinase with downstream functions in the IIS and target of rapamycin (TOR) cascades. *HR46* is an ecdysone-inducible nuclear hormone receptor that is involved in many processes including nervous system development. *TYR* shows tissue-specific differential expression in the brain, fat body, and ovaries of high- and low-strain bees.

Transcriptional Profiles of Worker Ovaries

Transcriptional patterns between high- and low-strain young-worker ovaries were compared (117) using an established microarray platform (36, 119) and qRT-PCR. Approximately 20% of the 10,586 transcripts in the microarray varied between workers of the high and low pollen-hoarding strains, including two of the positional candidate genes for *pln* QTLs, *TYR* and *HR46*. These genes were also differentially expressed in wild-type workers with more, and fewer, ovarioles. The

differentiation in oviarole number between high- and low-strain bees further supports the relationships between ovariole number and ovary expression of those candidate genes. *ftz-f1* is an additional gene of interest identified in the microarray study. *ftz-f1* and HR46 are both involved in the ecdysteroid signaling cascade and show correlated expression with ovariole number in pollen-hoarding strains as well as wild-type bees (116, 117).

The Function of Reproductive Gene Expression in Honeybee Social Behavior

QTL mapping and gene-expression studies using bees from the high and low strains and wild-type bees clearly demonstrate the links between reproduction and individual and social behavior. They also demonstrate that reproductive regulatory systems have been used by natural selection and by the pollen-hoarding artificial-selection program to shape the foraging division of labor in honeybees. As described above, candidate genes that warrant further functional investigation have emerged from these studies. In honeybees, adult gene expression can be suppressed by RNAi mediated by interabdominal injections of double-stranded RNA (dsRNA). The technique was first developed for long-interfering (>500 bp) RNA and later expanded to short-interfering RNA (49, 50). Initial experiments focused on *vitellogenin*, which encodes a yolk-protein precursor protein that is exclusively synthesized by the trophocyte cells of the fat body. Insect fat-body tissue is functionally homologous to the vertebrate liver and white fat and consists of trophocyte and oenocyte cells in honeybees. In *Drosophila melanogaster*, the oenocyte cells are central to lipid metabolism, whereas similar functional compartmentalization has not been verified in honeybees. Trophocytes and oenocytes, however, differ in dsRNA accessibility. Trophocytes can take up considerable amounts of dsRNA (49) that remains detectable several days after injection (9). Oenocytes take up significantly less dsRNA, and similar reduced uptake is found in ovary and brain tissue (49). The restrictions of honeybee cells and organs to dsRNA limit the functional testing of reproductive genes in honeybee behavior, but they also provide opportunities. For example, the genes that are expressed by trophocyte cells, such as *vitellogenin*, currently represent the most feasible targets for functional genetic research on honeybee behavior.

The insect brain contains critical control circuits of behavioral programs that are involved in reproduction (22). Although researchers are still presented with challenges when trying to manipulate such circuits in honeybees, they can employ functional genetics within peripheral cells and organs because these larger systems also affect the animal brain and behavior. The fat body, for example, produces yolk proteins and peptides that are essential for insect egg development: It stores nutrients that fuel oogenesis and reproductive activity, it partakes in nutrient sensing, and it communicates nutrient status to the brain (32). These peripheral processes are critical to reproduction and can be specifically targeted by intra-abdominal dsRNA because the resulting RNAi response will not suppress target genes in other tissues. RNAi-mediated gene knockdown in honeybees, in other words, provides unique opportunities for studying the roles of a peripheral (non-neural) tissue engaged in the "remote control" of behavior.

Vitellogenin. Knockdown of the *vitellogenin* gene provided the first functional genetic evidence for a role of fat-body cells in the regulation of social behavior (5, 37, 61). This role was proposed several years earlier by a theoretical model in which the Vg protein suppressed an endocrine factor, JH, that was believed to be central to foraging behavior. Vg and JH acted together in a feedback loop to control the timing of foraging onset in workers: High Vg levels suppressed JH and foraging behavior, whereas high JH levels suppressed Vg and nursing behavior (**Figure 2**). RNAi experiments confirmed that JH increased

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when *vitellogenin* was suppressed (37) and that *vitellogenin*-knockdown workers foraged precociously (58, 61). The *vitellogenin* knockdowns, however, also biased their foraging efforts toward nectar (61). This result established that a yolk precursor gene also affected the foraging preference of honeybee workers.

The effect of *vitellogenin* on the foraging preference of worker bees was explained by a theoretical framework of the reproductive ground-plan hypothesis. In solitary ancestors of honeybees, high Vg levels were connected to pollen feeding that fueled vitellogenesis and pollen hoarding that provided provisions for the growing larvae. The gene-regulatory network that ensured that Vg production and pollen hoarding were linked became co-opted during social evolution (**Figure 3**). Expression of *vitellogenin*, thereby, biases honeybee workers toward pollen foraging, whereas suppression of *vitellogenin* by RNAi results in a bias toward nectar. Nectar is also consumed by many insects during nonreproductive phases of their life cycle.

The connection between the expression level of *vitellogenin* and worker foraging bias was also confirmed in the high and low pollen-hoarding strains. Like *vitellogenin* gene knockdowns, bees with the low pollen-hoarding strain have low Vg levels and bias foraging toward nectar, whereas bees with the high pollen-hoarding strain have high Vg levels and bias foraging toward pollen (4). The *vitellogenin* expression differences between these strains are most pronounced during the first 15 days of life, but they are also detectible in foragers (K. Ihle, R.E. Page & G.V. Amdam, unpublished data). Similarly, RNAi-mediated gene knockdown reduces *vitellogenin* during the nurse stage as well as the forager stage of workers (K. Ihle, R.E. Page & G.V. Amdam, unpublished data). The functional genetic data and the results from pollen-hoarding strains, therefore, do not resolve whether the influence of Vg on foraging bias takes place in foragers or whether consistently low Vg levels are required during worker maturation.

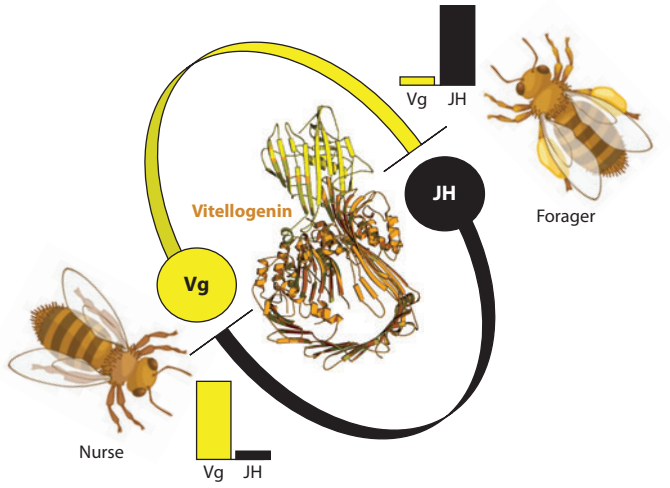

Figure 2

The honeybee vitellogenin (Vg) protein (*structural model in center*) suppresses juvenile hormone (JH) and forager behavior in worker bees. Nurse bees are high in Vg and low in JH. Nurses use Vg to produce proteinaceous jelly that is fed to larvae.

The cumulative data on *vitellogenin* demonstrate that a reproductive gene affects complex social behavior in worker honeybees by affecting the onset of foraging and foraging bias. Worker bees with both wild-type (unselected) and high pollen-hoarding strains respond to *vitellogenin* downregulation with precocious foraging and nectar collection. By contrast, bees with the low pollen-hoarding strain show no phenotypic response to *vitellogenin* RNAi: They do not forage precociously, and they do not collect more nectar (47). In accordance with this null response, low-strain bees also lack the JH increase that is induced by *vitellogenin* downregulation (3). This null-mutant genotype may, in combination with high-strain and wild-type bees, provide unique opportunities to study the Vg-JH feedback loop, as it appears to have been at least partly disabled by colony-level selection for low levels of pollen hoarding.

Target of rapamycin; ultraspiracle. TOR is a nutrient-sensing molecular machine that responds to amino acids (63). Insect Vg levels are sensitive to protein availability (15, 19),

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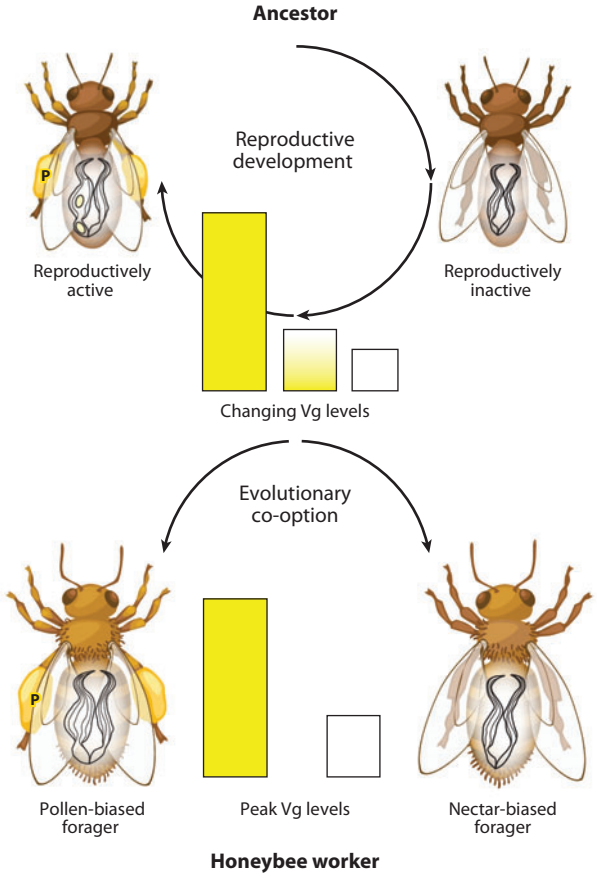

Figure 3

Female ancestors of honeybees went through a phase of reproductive inactivity with low vitellogenin (Vg) levels and a phase of reproductive activity with high Vg, vitellogenesis, and pollen hoarding. Pollen hoarding differs from pollen consumption. In hoarding, pollen is collected and stored in a nest as a protein source for larval development. Similarly, high *vitellogenin* gene expression is linked to pollen hoarding in honeybees.

and RNAi-mediated suppression of *TOR* reduces *vitellogenin* expression in honeybees and mosquitoes (80). RNAi to *vitellogenin*, moreover, does not affect *TOR*; therefore, *TOR* is upstream of *vitellogenin*. TOR signaling is central to reproduction in many insects and can affect JH levels (57, 59). Thus, *TOR* can provide another connection between reproductive-gene networks and worker honeybee behavior. Experiments with the drug rapamycin, a competitive inhibitor of TOR signaling, suggest that TOR may influence AFF (10). However, the studies were not conclusive. Foraging onset was both accelerated and retarded by rapamycin. Interactions between TOR signaling, other nutrient-sensing pathways, and environmental factors may explain these variable results (10), but more research is clearly needed to validate the connections.

In insects, *ultraspiracle* (*usp*) is an ecdysteroid receptor and putative JH receptor/response element that functions in development, growth, and reproduction (41). In combination with *vitellogenin* RNAi, RNAi-mediated knockdown of *usp* in honeybees results in highly elevated JH levels, an increase in gustatory responsiveness, reduced starvation resistance, and mobilization of sugars (trehalose and glucose) to the hemolymph (Y. Wang & G.V. Amdam, unpublished data). Ecdysteroid signaling via USP is believed to have little, if any, role in the behavioral regulation of eusocial insects because ecdysteroid levels are very low in the adults (38). The combined effect of *vitellogenin* and *usp* RNAi may instead be due to a compensatory increase in hormone (i.e., JH released by *vitellogenin* RNAi) that is often observed in mutants with deficient receptors. JH, moreover, does not increase when *usp* is suppressed in bees with normal Vg levels, supporting the hypothesis that *vitellogenin* controls JH (**Figure 4**). The effects of *vitellogenin* and *usp* double knockdown have not been tested for behavior other than gustatory responsiveness, which is a predictor of foraging bias (**Table 2**). Future experiments should test the Vg/*usp* connection to foraging preference as well as age at onset of foraging.

Insulin receptor substrate and other insulin-like signaling genes. An overabundance of IIS genes in the mapped pollen-hoarding QTLs suggests that this signaling network is affected by selection and influences behavior associated with the pollen-hoarding syndrome. The insulin/insulin signaling system can be essential to insect egg development (17), but more generally it guides resource allocation to reproduction during times of surplus and to somatic maintenance

during times of famine (108). *IRS* is a candidate gene for *pln4* and encodes the substrate protein that communicates signals via the insulin receptor and, therefore, is a central gene in the cascade. Insulin receptor substrate (IRS), however, also provides a substrate for other receptors, including the epidermal growth factor receptor. Effects of *IRS* downregulation in honeybees may, therefore, not be exclusively due to changes in insulin signaling (59).

RNAi-mediated suppression of fat-body *IRS* expression was performed in bees from both the high and low pollen-hoarding strains (118). In contrast to *vitellogenin* RNAi, which does not affect low-strain workers, both genotypes responded with reduced nectar collection coupled in high-strain knockdowns with an increase in pollen collection. Also unlike *vitellogenin* RNAi, the knockdown of *IRS* did not affect gustatory responsiveness or the *vitellogenin* mRNA level (118). An experiment on honeybee larvae also suggests that *IRS* downregulation can suppress JH (59), although this result has not been validated in adult bees. These studies demonstrate an influence of *IRS* on worker behavior that appears to be at least partly independent of the control circuit of *vitellogenin*.

The honeybee fat body also expresses genes for two putative ligands of the insulin receptor, insulin peptide 1 and 2 (ilp1, ilp2). Secretion of insulin peptides from the brain has been heavily studied in *Drosophila*, and this mechanism has been linked to regulation of reproduction and aging (31, 32). Less is known about the functions of insulin peptides that are produced by peripheral tissues. In honeybees, *ilp1* expression is confined to oenocytes, whereas *ilp2* is expressed in both oenocytes and trophocytes (**Figure 5**). Thus, it should be possible to test the role of *ilp2* in behavior.

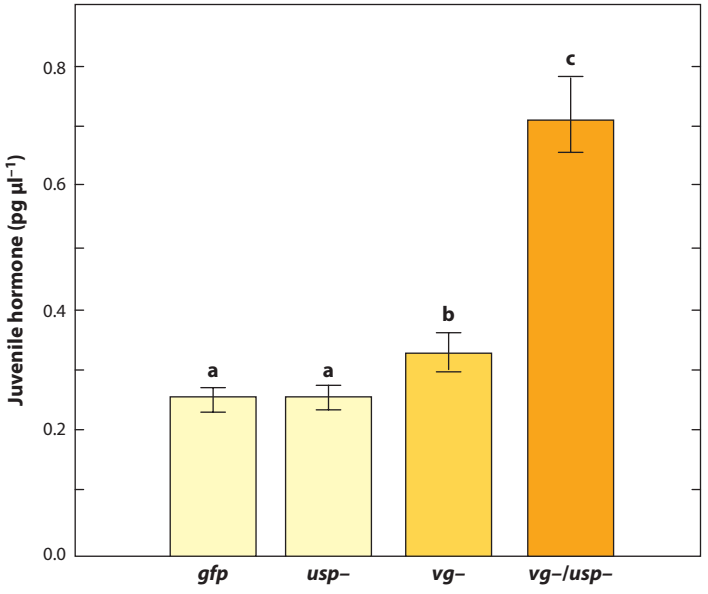

Figure 4

Juvenile hormone (JH) is increased by *vitellogenin* gene knockdown (*vg*-) relative to controls (*gfp*) and ultraspiracle knockdowns (*usp*-). JH levels in the blood increase more than twofold when *vg* and *usp* are suppressed together (*vg-/usp*-). Bars with different letters (*a*, *b*, *c*) are statistically different ($P < 0.05$).

SUMMARY

Two-way colony-level selection on a single social trait—the amount of pollen stored in the comb—resulted in changes at different levels of biological organization constituting a complex phenotypic architecture that we call the pollen-hoarding syndrome. Genetic mapping has demonstrated that the phenotypic architecture is derived from a complex epistatic and pleiotropic genetic network with effects on the reproductive regulation of honeybees. Honeybee sequence data of mapped QTLs reveal candidate genes that are differentially expressed in bees from the artificially selected strains and between wild-type bees that vary in phenotypes that define the pollen-hoarding syndrome. Gene-knockdown studies of candidate genes, using RNAi, confirm the effects of some of the candidate genes on behavior and other elements of the reproductive regulatory network and signaling networks closely associated with reproduction and development. Selection at the colony level for a social trait also left its signature on the hormonal control of ovary development that takes place in worker larvae. The work presented here represents the only detailed study of the genetics and developmental evolution of complex social organization.

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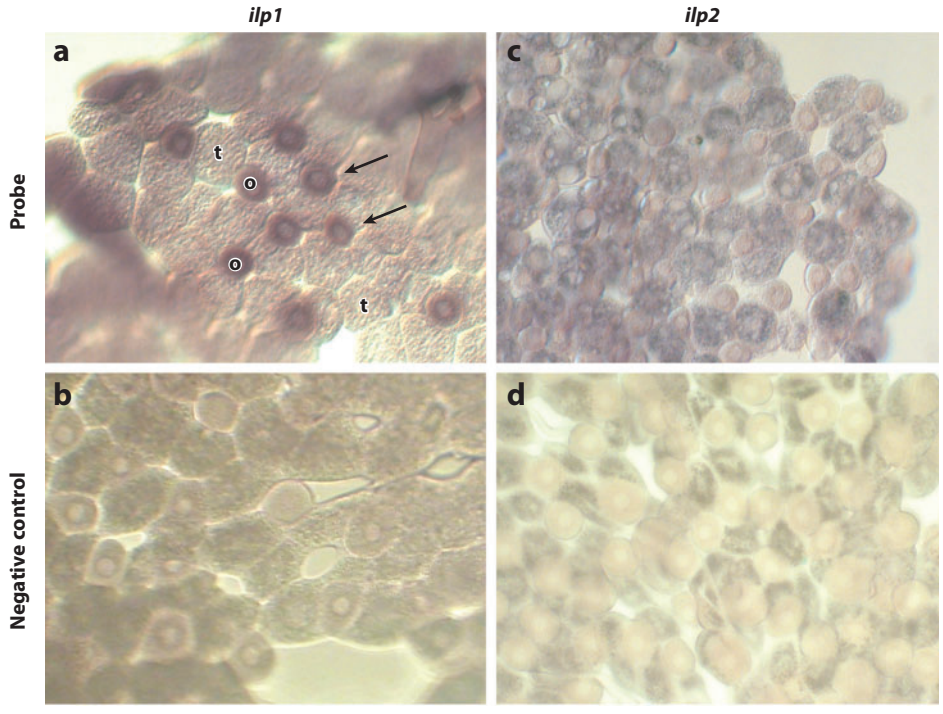

Figure 5

Insulin peptide (ilp) mRNA in honeybee worker fat-body cells stained by in situ hybridization. (*a*) *ilp1* is expressed in oenocytes (o), whereas (*c*) *ilp2* is expressed in both oenocyte and trophocyte (t) cells. (*b*,*d*) Negative controls. From Reference 62.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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